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Glycosaminoglycan Synthesis in Explants Derived from Bleomycin-Treated Fibrotic Hamster Lungs[1] (41657)

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Abstract. Glycosaminoglycan synthesis was studied in explant cultures of hamster lungs 15 and 45 days following intratracheal administration of Bleomycin. At both time points, a statistically significant increase in ^{35}S-sulfate incorporation into glycosaminoglycans was seen in the Bleomycin-treated explants compared with that of the controls. Furthermore, the percentage of label associated with dermatan sulfate was significantly higher in the treated explants than in controls at both 15 and 45 days. Conversely, the percentage of labeled heparin and/or heparan sulfate was significantly lower for the treated explants compared to controls at these times. These results indicate that glycosaminoglycan synthesis is altered from normal in this model of interstitial lung disease. Comparison of these data with previous measurements of glycosaminoglycan synthesis in another model of interstitial lung disease, induced by *N*-nitroso-*N*-methylurethane, reveals marked similarity in the changes from normal in ^{35}S-labeling.

The role of alterations in connective tissue components in the pathogenesis of interstitial pulmonary fibrosis remains poorly understood. This is especially true of pulmonary glycosaminoglycans. A number of studies have implicated these molecules in the deposition and organization of other connective tissue components, most especially collagen (1–4). In addition to possible regulatory functions in repair, glycosaminoglycans may also provide an essential structural tissue component in the restoration of damaged lung parenchyma. Nevertheless, only a small number of studies (5–7) have been published regarding changes from normal in the content and distribution of glycosaminoglycans in interstitial lung disease.

Recently, we reported the results of experiments measuring synthesis of glycosaminoglycans in a hamster model of interstitial pulmonary fibrosis induced by sequential injections of *N*-nitroso-*N*-methylurethane (NNNMU) (6). That study demonstrated changes from normal in the synthesis of glycosaminoglycans in animals with interstitial lung fibrosis induced by this agent. It remains to be determined whether alterations in glycosaminoglycan synthesis in the NNNMU model are specific for that agent or are similarly observed in other models of the disease induced by other chemical agents. The present study was undertaken to address this question by examining glycosaminoglycan synthesis in a second model of lung fibrosis, induced by intratracheal injection of bleomycin (8, 9). In this study, the effects on glycosaminoglycan synthesis of bleomycin injury were measured in lung explant culture and the results compared with previously determined measurements *in vivo* in the NNNMU model.

Materials and Methods. A total of 13 outbred, female Syrian hamsters (7 experimental, 6 control), weighing approximately 120 g each, were utilized in this study.

Induction of interstitial pulmonary fibrosis. Experimental animals received a single intratracheal injection of 1.0 mg (1.0 unit) bleomycin sulfate (Bristol Laboratories, Syracuse, N.Y.) dissolved in 0.2 ml sterile isotonic saline, while under light anesthesia, induced with 0.05 ml Ketaset (Bristol Laboratories, Syracuse, N.Y.) administered im. Controls, similarly anesthetized, received an intratracheal injection of 0.2 ml isotonic saline.

Preparation of lung tissues for explant culture. At 15 and 45 days following bleomycin instillation, both experimental and control animals were sacrificed by ip injection of chloral hydrate. The lung tissues were removed from the thorax, dissected free of extraparenchymal structures, and placed in Ham's F-12 medium. Portions of the lungs

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were minced and multiple 1 to 2-mm fragments were placed on filter paper-covered wire rafts, then suspended in the same medium, supplemented with 10% fetal calf serum, to which had been added ^{35}S-sodium sulfate (New England Nuclear, Boston, Mass.) to a concentration of 2 μCi/ml.

Labeling and measurement of glycosaminoglycans. The explants were labeled with ^{35}S-sulfate for 6 hr in an atmosphere of 5% CO_2, 95% air at 37°C, producing measurable, reproducible incorporation of the label into tissue. The explants were then frozen to terminate metabolic activity. Subsequently, the tissues were thawed, washed in normal saline, desiccated, and weighed. The samples were then worked up as previously described (6, 10). Briefly, the procedure used is as follows: Following delipidation in acetone, the glycosaminoglycans were isolated by papain digestion, TCA precipitation of undigested proteins, dialysis of the supernatants, and ethanol precipitation of the glycosaminoglycans in the presence of carrier chondroitin sulfate. After measurement of total labeling of the glycosaminoglycan fraction, aliquots of the material were degraded with either chondroitinase ABC or AC-II (Seikagaku Kogyo Co., Tokyo), according to the method of Saito *et al.* (11). The properties of these enzymes have previously been described (11–13). Following digestion, the samples were subjected to paper chromatography in a system of butanol:glacial acetic acid:1 *N* ammonia (2:3:1) This yielded several labeled constituent pools: (i) chondroitinase-resistant material, predominantly heparin and/or heparan sulfate, which remains at the origin; (ii) disaccharides of chondroitin 6-sulfate; (iii) disaccharides of chondroitin 4-sulfate and dermatan sulfate (with chondroitinase AC-II, dermatan sulfate remains undigested and chromatographs with heparin and/or heparan sulfate). The developed chromatograms were cut into uniform strips and assayed for radioactivity, after immersion in scintillation fluid, in a liquid scintillation spectrometer. By this method, it was possible to determine the percentage of label associated with: (i) heparin and/or heparan sulfate, (ii) chondroitin 6-sulfate, (iii) chondroitin 4-sulfate, and (iv) dermatan sulfate.

In all cases, the relatively short half life of ^{35}S (87 days) was accounted for by calculating the decay factor into the resultant data.

Histology. Representative fragments of tissue from the explant minces were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or hematoxylin–phloxine–safran. The slide sections were examined with the light microscope for evidence of interstitial disease.

Results. *Histology.* Light microscopic examination of the explant minces at 15 days postbleomycin showed a patchy disease process consisting of mononuclear cell infiltrates, alveolar epithelial hyperplasia, scattered fibroblast activity, and marked deposition of both fibrillar and amorphous extracellular material (Fig. 1). While the amount of disease varied among the explants, virtually all showed evidence of tissue reaction, including fibrosis.

At 45 days, a similar pattern of mononuclear cell and fibroblast infiltration, alveolar epithelial hyperplasia, and fibrosis was noted. In the reactive areas, the fibrosis was more marked than at 15 days.

Biochemistry. At 15 days postbleomycin, total incorporation of ^{35}S-sulfate into the experimental tissue was significantly higher ($P = 0.001$) than for controls (Table I). Again, at 45 days following initiation of disease, the bleomycin-treated explants had a significantly greater ($P = 0.02$) amount of labeled glycosaminoglycans, though the difference between the groups was less than at 15 days (Table I).

As mentioned in Methods, the data presented are independent of any ^{35}S decay occurring in the sample groups.

With regard to ^{35}S uptake by the various subgroups of glycosaminoglycans, significant differences between the experimental and control groups were observed at both 15 and 45 days postbleomycin. At 15 days, the treated explants had a statistically significantly greater ($P = 0.02$) percentage of labeled dermatan sulfate than did controls (21.4% vs 13.3%). Conversely, the experimental tissues had a significantly lower ($P = 0.006$) percentage of chondroitinase-resistant material (heparin and/or heparan sulfate) than did the corresponding controls (38.2% vs 50.9%). The two groups had similar percentages of labeled chondroitin 4-sulfate and chondroitin 6-sulfate (Table II).

At 45 days postbleomycin, the labeling differences between experimental and control explants were similar to those at 15 days. The bleomycin-treated explants again had a sig-

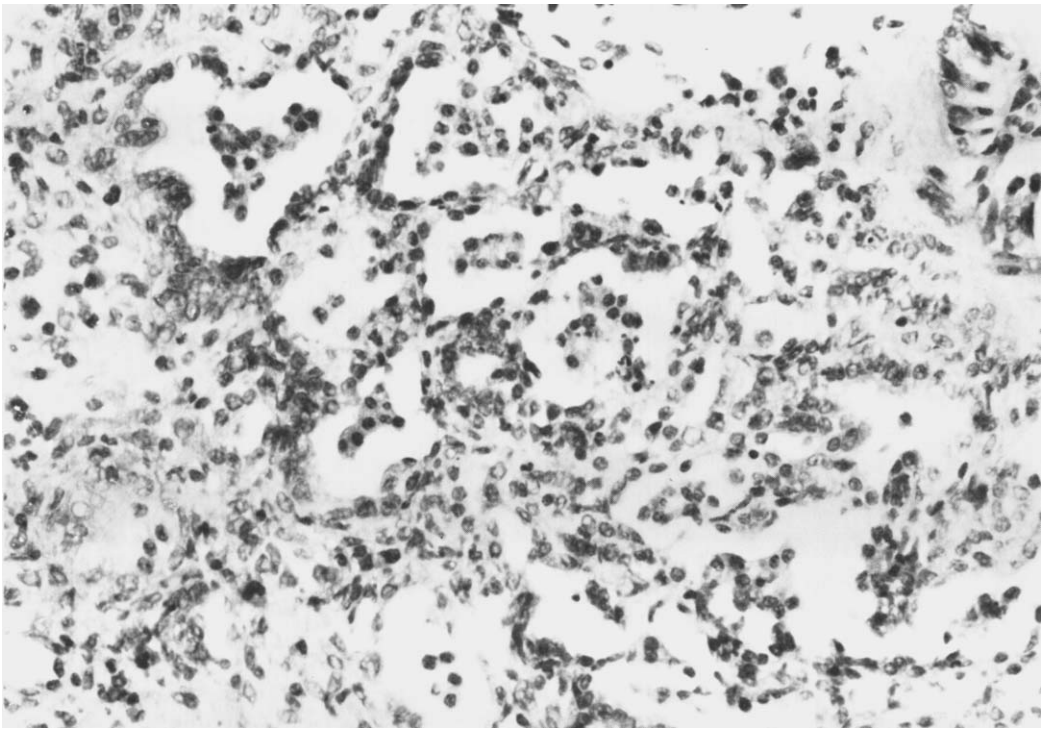

FIG. 1. Photomicrograph of an explant derived from a hamster lung 15 days post-intratracheal instillation of bleomycin. Interstitial thickening and fibrosis is associated with alveolar epithelial hyperplasia, scattered fibroblast activity, and mononuclear cell infiltrates. Hematoxylin–phloxine–safran, ×292.

nificantly greater ($P = 0.003$) percentage of labeled dermatan sulfate than did controls (17.7% vs 10.8%). The treated explants also showed a significant decrease ($P = 0.004$) in the percentage of labeled heparin and/or heparan sulfate compared to controls (52.6% vs 61.9%). As at 15 days, the percentage of label associated with chondroitin 4-sulfate and chondroitin 6-sulfate was similar for the two groups (Table II).

Since it may be argued that multiple explants from a single animal are not truly independent samples, the data were additionally analyzed for similar group differences by arranging them according to animal. Despite the fewer degrees of freedom imposed by this analysis, statistical significance ($P \leq 0.05$; one-tailed t test) was seen between experimental and control animals in regard to: (i) total ^{35}S incorporation at 45 days; (ii) percentage of labeled dermatan sulfate at 15 and 45 days; (iii) percentage of labeled heparin and/or heparan sulfate at 15 and 45 days. Regarding total ^{35}S uptake at 15 days, the P value was slightly higher (0.06), reflecting the large variance in the experimental group.

Discussion. This study provides evidence that the repair process following intratracheal instillation of bleomycin into the lungs of hamsters is associated with significant changes

TABLE I. TOTAL INCORPORATION OF ^{35}S-SULFATE INTO LUNG GLYCOSAMINOGLYCANS

Group	Day postbleo	CPM/mg dry lung wt
Exp (14)	15	2936 ± 433*
Cont (7)		1099 ± 131*
Exp (12)	45	1461 ± 85**
Cont (8)		1209 ± 50**

Note. Values expressed as mean ± SE. Figures in parentheses refer to number of explants tested. Explants were derived from individual experimental animals (E1, E2, etc.) and controls (C1, C2 etc.) in the following numbers: 15 days postbleomycin: E1:4, E2:4, E3:4, E4:2, C1:4, C2:2, C3:1; 45 days postbleomycin: E1:4, E2:4, E3:4, C1:4, C2:2, C3:2.

* $p \leq 0.001$ (two-tailed t test).

** $p \leq 0.05$ (two-tailed t test).

TABLE II. PERCENTAGE DISTRIBUTION OF ^{35}S AMONG LUNG GLYCOSAMINOGLYCANS

Group	Days postbleo	Heparin/heparan S[a]	Ch4-S	Ch6-S	Derm S
Exp (14)	15	38.2 ± 1.1**	24.8 ± 1.3	15.6 ± 1.2	21.4 ± 1.7*
Cont (7)		50.9 ± 3.1**	22.7 ± 3.2	13.1 ± 1.3	13.3 ± 3.1*
Exp (12)	45	52.6 ± 1.1**	18.5 ± 1.0	11.2 ± 0.5	17.7 ± 1.4**
Cont (8)		61.9 ± 2.3**	17.7 ± 1.0	9.6 ± 1.3	10.8 ± 1.3**

Note. Values expressed as mean ± SE. Figures in parentheses refer to number of explants tested.
[a] Chondroitinase ABC-resistant material (see Methods).
* $p \leq 0.05$ (two-tailed t test).
** $p \leq 0.01$ (two-tailed t test).

from control in the synthesis of glycosaminoglycans. An overall increase in total ^{35}S-sulfate incorporation was observed in the treated explants at both 15 and 45 days postbleomycin as well as specific increases in the relative percentage of labeled dermatan sulfate and decreases in the proportion of label associated with heparin and/or heparan sulfate.

These findings are similar to those obtained with the NNNMU model of pulmonary fibrosis (6) previously studied in this laboratory. With that model, a significant increase in total uptake of ^{35}S into glycosaminoglycans was observed compared to controls along with increases in the percentage of labeled dermatan sulfate and decreases in the percentage of labeled heparin and/or heparan sulfate. This similarity in glycosaminoglycan labeling suggests that the parenchymal tissue response to injury induced by these different agents is qualitatively similar despite differences between the models. In the NNNMU model, up to 16 subcutaneous injections were given as compared to a single intratracheal insufflation in the bleomycin model. Also, glycosaminoglycans were measured in the NNNMU model at 1 and 3 months following completion of the injection schedule in contrast to 15 and 45 days postadministration of Bleomycin.

Recently completed *in vivo* measurements (unpublished data) of glycosaminoglycan labeling in the bleomycin model are consistent with the recent findings observed in explant cultures of bleomycin-treated lungs and indicate the usefulness of the explant technique in these types of studies. An advantage of the explant system is that it fixes the extracellular pool size of ^{35}S-sulfate, thereby minimizing differences in labeling of glycosaminoglycans imposed by variations in the specific radioactivity of the sulfate in the lung tissue of experimental and control animals.

The etiology of the observed changes from normal in glycosaminoglycan labeling in the bleomycin and NNNMU models is not understood. Little is known about glycosaminoglycan synthesis by individual lung cell types, both normally and following injury. The changes in the types of cells which populate the lung after this experimental injury may be responsible for at least some of the findings. In the bleomycin model, there is an initial, variable, acute inflammatory response lasting several days, followed by rapid regeneration of alveolar epithelium, migration of fibroblasts into the areas of injury, and fibrosis of the parenchyma, manifested both histologically and biochemically by 2 weeks (9, 14). These cellular changes may also be accompanied by alterations in the glycosaminoglycan-synthesizing capacities of one or more cell types, resulting perhaps from a change in cellular differentiation or from enhanced proliferation, subsequent to injury. In addition to altered rates of synthesis of glycosaminoglycans following lung injury, changes in the rate of degradation may also affect the pattern of labeling. Moreover, changes from normal in either the chain length of the glycosaminoglycans or in their level of sulfation would also influence ^{35}S incorporation. The hypothesis that actual increases in glycosaminoglycan synthesis occur is supported by a recent paper by Karlinsky (7) which shows that total glycosaminoglycan content in the bleomycin-treated hamster lung is elevated significantly from control one month after bleomycin administration.

Regarding possible roles for glycosaminoglycans in the lung repair process, there is already evidence (1) that synthesis of this con-

nective tissue component is consistently elevated in tissues undergoing repair from several forms of injury in organs other than the lung. Furthermore, glycosaminoglycans have been shown to influence the deposition and degradation of collagen fibers (1). Other studies suggest that glycosaminoglycans may stimulate the proliferation of fibroblasts and help control collagen synthesis by these cells (15, 16).

In the intratracheal bleomycin model, collagen synthesis is increased from normal beginning at 6 days postinstillation of the agent (14) and continuing for 2 weeks thereafter, encompassing the 15-day increase in glycosaminoglycan synthesis. Furthermore, the marked drop in total ^{35}S incorporation between 15 and 45 days coincides with a similar decline in collagen synthesis reported in this model (14). This provides some circumstantial evidence of a possible link between the synthesis of these two connective tissue components which needs further investigation. Earlier studies of glycosaminoglycan synthesis in this model would be useful to determine whether the initial elevations in this connective tissue component coincide with those of collagen. The precise relationship between glycosaminoglycans and collagen synthesis and degradation in the lung must be elucidated before an understanding of the role of connective tissue abnormalities in the development of interstitial pulmonary fibrosis is obtained. The consistent increases in labeled dermatan sulfate found in both the NNNMU and bleomycin models of the disease suggest a specific role for this molecule in the fibrotic response of the lung.

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